EVIDENCE FOR MULTIPLE GENE CONTROL OF A SINGLE POLYPEPTIDE CHAIN: THE HEAVY CHAIN OF RABBIT IMMUNOGLOBULIN

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Abstract.—To determine the chemical basis for rabbit heavy chain allotypes, amino acid analyses were carried out on IgG and IgM antibodies isolated from rabbits which were homozygous a1 or a3 for the γ chain locus and homozygous b4 for the light chain locus. The compositional differences between a1 and a3 IgM antibodies were found to be identical to those between their IgG counterparts. The identity of the allotypic amino acid replacements showed that the same genetic markers were present in the variable sequences of IgG and IgM heavy chains. Since the constant sequences of these heavy chains are controlled by different loci, these data demonstrated that rabbit heavy chains are coded by two separate germ-line genes, one producing the variable region and a second one of various genes producing the constant regions.

The use of two antibodies, antiazophenylarsonate and antiazophenyl-β-lactoside, permitted the compositions to be compared also on the basis of immunological specificity. The specificity amino acid replacements were found to be identical in the IgM and IgG heavy chains whether the two antibodies were isolated from an individual animal of a1 or a3 allotype. These results further support the conclusions of the allotype measurements that at least two genes code for the rabbit heavy chain.

The light and heavy polypeptide chains of the immunoglobulins consist of a C-terminal region of constant sequence and an N-terminal region of variable sequence.[1, 2] There is general agreement that the constant sequence of each chain class is determined by a single gene carried in the germ line, i.e., genes C_κ and C_λ for the light chain classes, kappa and lambda, and genes C_γ, C_α, and C_μ for the three heavy chains which define the major classes of immunoglobulins, IgG, IgA, and IgM. The question not yet answered is whether the variable sequences are determined by multiple genes in the germ line or by one or a few genes from which many are created during the somatic development of the immune system.

Evidence to help resolve this question was obtained in our laboratory[3] from the compositional analyses of peptides which were isolated from the heavy chains of rabbit IgG antibodies. Both the amino acid replacements corresponding to the genetic markers and those corresponding to the antibody specificity were located in the N-terminal portion of the γ chain; the allotypic differences were concentrated for the most part in the first 34 residues of the variable region while the specificity differences were found to be distributed between residues 35 and 180. These results provided a strong argument against multiple V-genes in the germ

line[4] since it was improbable that each of the genes would evolve with the same information for the genetic markers. The data were more consistent with the somatic theories for antibody diversity although restrictions would be required of any of the proposed mechanism (recombination,[5] mutation,[6] and repair[7]) to explain the maintenance of the genetic markers in variable sequences.

To obtain additional evidence, our investigations have been extended to rabbit IgA and IgM antibodies. Their α and μ chains have been shown by Todd and others[8-10] to react with antisera specific for the allotypic determinants of the rabbit γ chains. The purpose of the present experiments was, therefore, to compare the amino acid replacements associated with allotypic specificity in the γ, α, and μ chains and determine whether identical or cross-reacting genetic markers are present. If the allotypic amino acid replacements are found to be identical in each chain, the paradox results that allelic genetic markers in the variable region are associated with constant regions produced by the nonallelic genes, C_γ, C_α, and C_μ. This paradox can be resolved by postulating that a single structural gene ($V_{(H)}$) controls the variable region and is shared by all classes of heavy chain ($C_{(H)}$).[11] If, on the other hand, the allotypic amino acids are similar but not identical in each chain, three structural genes, V_γ, V_α, and V_μ, control the variable regions of their respective heavy chains.

The present paper describes the first part of these investigations, the comparison of the allotypic and specificity amino acid differences in rabbit IgM and IgG. Specific antibodies were chosen rather than nonspecific immunoglobulin fractions because (1) the purification of IgM was facilitated and (2) the concurrent analyses of the amino acids associated with antibody specificity provided a further test of the alternative mechanisms of heavy chain synthesis. Rabbits which were homozygous a1 or a3[12, 13] for the γ chain locus and homozygous b4 for the light chain locus were immunized against the determinants previously studied, phenylarsonic acid and phenyl-β-lactoside coupled to a bovine γ globulin carrier. The IgM and IgG fractions of both antibodies were isolated from individual animals and their compositions determined. Since the light chains were known to be common to both classes of immunoglobulins, the relevant heavy chain differences could be determined from analyses of the molecules.

Experimental Methods.—Immunization of rabbits: The immunizing antigens consisted of *p*-aminophenylarsonic acid and *p*-aminophenyl-β-lactoside coupled to bovine γ globulin according to procedures previously described.[14] The azoantigens were coprecipitated with alum, and the mixture was injected intravenously over a period of 4 weeks into New Zealand white rabbits whose genotype with respect to the *a* and *b* loci was $a^1a^1b^4b^4$ or $a^3a^3b^4b^4$. Each animal received a total 120 mg of antigen and was bled out 4 days after the last injection.

Purification of IgM and IgG antibodies: The arsonic and lac[15] antibodies were isolated from the serum of individual animals using the bromoacetyl cellulose immunoadsorbent (BAC) developed by Robbins *et al.*[16, 17] The specific antigen cellulose conjugates were prepared by coupling 200 mg of *p*-azophenylarsenate bovine serum albumin or *p*-azophenyl-β-lactoside bovine serum albumin to 1 gm of BAC. The antiserum was then mixed with the appropriate BAC for 1 hr in the cold at a ratio of 20 ml of serum to 0.1 gm of resin. The antibody was dissociated from the immunoadsorbent by incubating with hapten for 15 min at 37° at a ratio of 2 ml of hapten to 0.1 gm of resin. The haptens used were 0.3 *M* sodium phenylarsenate and 0.5 *M* lactose adjusted to pH 7.5. The IgM and

IgG fractions of each antibody were separated by chromatography on a column (2.2 × 100 cm) of Sephadex G-200 equilibrated with 0.5 *M* NaCl, 0.02 *M* Tris buffer, pH 8.0, and 0.001 *M* ethylenediaminetetraacetic acid (EDTA).

Amino acid analyses: Analyses were carried out on the dried, desalted eluates from the Sephadex G-200 fractionation according to the standard method.[18] The results were normalized to leucine contents of 89 and 98 residues, respectively, for the IgG and IgM antibodies. The value of 89 was based on previous measurements[16] of the average moles of leucine per mole of arsonic IgG; the value of 98 was calculated from the average per cent leucine yield on the IgM analyses and the assumption that the IgM monomeric unit contained the same total number of residues as the IgG antibodies.

TABLE 1. *Yields of antihapten IgM and IgG antibodies.*

Antibody	IgM* (mg/ml)	IgG* (mg/ml)	IgM (%)
Arsonic	0.028 ± 0.007†	0.21 ± 0.037	11.2 ± 1.1
Lac	0.039 ± 0.005	0.21 ± 0.044	17.5 ± 2.0

* Average yield from 6 animals.
† Standard error of the mean.

Results.—The IgM and IgG responses to the hapten determinants are summarized in Table 1. Despite the considerable variation in yields among the individual sera the average arsonic and lac responses were very similar. The only exception was the consistently higher per cent of lac antibody in the form of IgM, 17.5 per cent compared to 11.2 per cent for the arsonic IgM. This finding agrees with other reports that carbohydrate determinants elicit a proportionately higher IgM response.[19, 20]

Since the purity of the antibody preparations was critical to the evaluation of the results, it was examined by several different methods. First, the binding capacity of the IgG antibodies was measured by equilibrium dialysis, using ^{14}C-labeled acetylated sodium arsanilate or ^{14}C-labeled lactose. Of a theoretical maximum of 2 moles of hapten per mole of antibody, the arsonic IgG bound an average of 1.92 and the lac IgG an average of 1.96. These values indicated that preparations were pure within the limits of detection by the dialysis method. The yields of IgM antibodies from individual sera, 1.0–3.5 mg, were not sufficient for binding measurements.

The antibody preparations were also tested by immunoelectrophoresis against a sheep antiserum to whole rabbit serum. At a concentration of 1 mg/ml, the IgG and IgM antibodies showed no other arc than the one corresponding to the respective IgG or IgM. At a concentration of 10 mg/ml, the IgG preparations again exhibited only a single arc, but the IgM preparations gave a faint precipitin line in the IgG region. The amounts of the IgG contaminants were determined by diluting the IgM antibodies and a control IgG antibody until precipitin arcs were no longer detectable. From these measurements, the IgG aggregates were found to comprise more than 2 per cent and less than 4 per cent of the IgM preparations.

The results of amino acid analyses on the 20-hour hydrolysates of the a1b4 antibodies are presented in Table 2 and those of the a3b4 antibodies are presented in Table 3. The data in Table 2 represent the average yields of four preparations, each isolated from individual animals, while the data in Table 3 represent the

TABLE 2. *Comparison of the amino acid compositions* of arsonic and lac (1) IgM and (2) IgG antibodies isolated from a1b4 rabbits.*

	IgM		IgG	
	Arsonic	Lac	Arsonic	Lac
Lys	63.4 ± 1.3†	63.0 ± 0.60†	70.4 ± 0.28	70.1 ± 0.20
His	23.1 ± 0.51	23.2 ± 0.35	16.1 ± 0.072	16.7 ± 0.10
Arg	49.3 ± 0.51	49.6 ± 0.38	45.3 ± 0.21	45.3 ± 0.11
Asp	107 ± 0.10	113 ± 0.35	107 ± 0.23	113 ± 0.25
Thr	151 ± 0.72	151 ± 0.46	170 ± 0.57	169 ± 0.53
Ser	157 ± 0.80	150 ± 0.72	156 ± 1.2	148 ± 0.88
Glu	126 ± 1.3	126 ± 1.3	124 ± 0.45	123 ± 0.74
Pro	92.6 ± 0.45	92.2 ± 1.2	112 ± 0.52	112 ± 0.16
Gly	109 ± 2.9	107 ± 1.8	112 ± 0.52	110 ± 0.29
Ala	89.0 ± 0.35	86.5 ± 0.30	78.7 ± 0.27	76.3 ± 0.62
Val	121 ± 1.4	122 ± 0.73	130 ± 0.47	132 ± 0.47
Met	14.1 ± 0.71	13.6 ± 0.26	12.8 ± 0.27	13.2 ± 0.65
Ileu	46.8 ± 0.30	45.8 ± 0.56	47.0 ± 0.07	46.3 ± 0.30
Leu	98	98	89	89
Tyr	46.7 ± 0.63	40.7 ± 0.31	56.0 ± 0.43	49.7 ± 0.25
Phe	53.4 ± 0.16	53.8 ± 0.20	44.8 ± 0.22	45.1 ± 0.16

* After 20-hr hydrolysis. The threonine, serine, tyrosine, and valine values represent 95.5, 90.1, 99.1, and 95.0 recoveries, respectively, as determined from additional 72-hr hydrolysis.
† Standard error of the mean.

average of three. The procedures used in the presentation of the results and in the statistical analyses have been described in detail elsewhere.[18]

The compositions obtained showed that the antibody preparations in these experiments were typical for the method of immunization and the antigenic determinants used. For example, the average compositions of the arsonic and lac IgG antibodies in Table 2 were indistinguishable from the values obtained in previous studies[14] with a large series of a1b4 rabbits, and the composition of the

TABLE 3. *Comparison of the amino acid compositions* of arsonic and lac (1) IgM and (2) IgG antibodies isolated from a3b4 rabbits.*

	IgM		IgG	
	Arsonic	Lac	Arsonic	Lac
Lys	64.1 ± 1.3†	64.3 ± 0.08	69.9 ± 0.37	69.8 ± 1.4
His	22.3 ± 0.20	22.9 ± 1.0	16.1 ± 0.27	16.5 ± 0.20
Arg	47.1 ± 0	47.3 ± 0.07	43.3 ± 0.44	42.9 ± 0.20
Asp	106 ± 0.47	111 ± 0.10	105 ± 0.45	111 ± 0.89
Thr	145 ± 0.18	145 ± 1.5	164 ± 0.44	162 ± 0.80
Ser	155 ± 1.6	148 ± 2.1	155 ± 0.04	148 ± 1.3
Glu	126 ± 0.10	126 ± 1.7	124 ± 0.67	123 ± 0.40
Pro	90.5 ± 1.0	89.8 ± 0.61	109 ± 0.47	109 ± 1.3
Gly	109 ± 0.51	108 ± 2.0	110 ± 0.35	110 ± 0
Ala	93.2 ± 0.10	90.4 ± 0	83.6 ± 0.70	80.9 ± 0.50
Val	117 ± 0.49	119 ± 1.5	127 ± 0.41	129 ± 0.75
Met	14.4 ± 1.0	...	14.4 ± 0.05	14.3 ± 0
Ileu	44.6 ± 0.56	44.0 ± 0.10	44.7 ± 0.41	44.2 ± 0.55
Leu	98	98	89	89
Tyr	46.7 ± 0.63	40.2 ± 0.51	55.7 ± 0.30	49.5 ± 0.55
Phe	55.7 ± 0.63	56.1 ± 0.16	47.2 ± 0.12	47.3 ± 0.35

* After 20-hr hydrolysis. The threonine, serine, tyrosine, and valine values represent 95.5, 90.1, 99.1, and 95.0 recoveries, respectively, as determined from additional 72-hr hydrolysis.
† Standard error of the mean.

arsonic IgM was in good agreement with that of the single rabbit IgM antibody previously reported.[21]

The results shown in Table 4 were obtained when the compositions were compared on the basis of allotypic and antibody specificities. In these comparisons, a difference in the content in any one amino acid was considered significant if the value exceeded three times the standard error of the mean of the difference. The choice of this standard meant that the minimum confidence level of the differences listed in the table was 98 per cent, and most of the differences were significant at much higher confidence levels. The few values within parentheses represent differences which did not meet these significance criteria but were included in the table to indicate a similar trend in the results.

It is evident from the allotypic comparisons that the compositional differences between the a1 and a3 IgM antibodies were identical to those between the a1 and a3 IgG antibodies. These results correlated well with previous measurements[3] of a1 and a3 Fd fragments from rabbit IgG antibodies; the differences involved the same residues and were twice the magnitude as would be predicted from the presence of two Fd fragments per IgG or IgM monomer. Thus, within the limits of detection of the method, the same allotypic amino acid replacements were found in the μ and γ chains. Final confirmation of these results will depend on the isolation of sufficient IgM antibodies to ascertain the location of these replacements in the variable region peptides.

The antibodies in these experiments were obtained from rabbits which were homozygous with respect to their $V_{(H)}$ gene, but of unknown phenotype with respect to their $C_{(H)}$ genes. Since allotypic specificities (a8–a12) have been identified with the constant region of γ chains[22–24] and (m1–m8) with the constant region of μ chains,[25] changes in amino acid content associated with polymorphism of the $C_{(H)}$ genes were expected. However, no significant differences in composition were observed among the IgG or IgM antibodies from individual

TABLE 4. *Differences in the amino acid compositions of rabbit IgM and IgG antibodies associated with (1) allotypic and (2) immunological specificity.*

	Allotypic Difference (a3 − a1)				Specificity Difference (Lac-arsonic)			
	IgM		IgG		IgM		IgG	
	Arsonic	Lac	Arsonic	Lac	a1	a3	a1	a3
Lys								
His								
Arg	−2.2	−2.3	−2.0	−2.4				
Asp	(−1)	−2	−2	(−2)	+6	+5	+6	+6
Thr	−6	−6	−6	−7				
Ser					−7	−7	−8	−7
Glu								
Pro	(−2.1)	(−2.4)	−3	(−3)				
Gly								
Ala	+4.1	+3.8	+4.9	+4.6	−2.5	−2.8	−2.4	−2.7
Val	−4	(−3)	−3	−3	(+1)	(+2)	+2	(+2)
Met								
Ileu	−2.2	−1.8	−2.3	−2.1				
Leu								
Tyr					−6.0	−6.5	−6.3	−6.2
Phe	+2.3	+2.3	+2.4	+2.2				

animals which could be correlated with C_γ or C_μ allotypes. There are several possible explanations for the negative results obtained: the allelic products of the $C_{(H)}$ genes may differ by so few residues that the replacements could not be detected, or linkage between alleles of the $V_{(H)}$ gene and alleles of any $C_{(H)}$ gene may be restricted as the data of Hamers and Hamers-Casterman[22] suggest. For example, all the a3b4 breeding stock used in these experiments were found to be a11 negative and a12 positive.

It is evident from the specificity comparisons in Table 4 that the amino acid changes between arsonic and lac IgM were precisely those observed between their IgG counterparts. The aspartic acid, alanine, valine, and two of the six tyrosine differences were previously found to be located in the arsonic and lac light chains,[14] and since the light chains are common to all classes of immunoglobulins, these differences were expected in a comparison of the IgM compositions. However, the serine and the remaining four tyrosine differences were known to be located in the γ chains of the IgG antibodies[14] and thus their appearance in the IgM compositions demonstrated that the specificity amino acid replacements were the same in the μ as in the γ chains. Furthermore, the finding of the characteristic serine and tyrosine differences whether the arsonic and lac antibodies were isolated from a1 or a3 rabbits showed that the specificity amino acid replacements were independent of the allotype present in the variable region.

Discussion.—It has been generally accepted from the data on enzyme biosynthesis that a single cistron controls a single polypeptide chain. However, the data on antibody sequences and their genetic control have increasingly led to the speculation that the synthesis of antibody chains may involve a different genetic mechanism. In the present work, the identity of the allotypic amino acid replacements in IgG and IgM demonstrates that the same genetic markers are present on the γ, μ, and by inference, the α chains of rabbit immunoglobulins. Since these allotypic markers are known to distinguish the allelic products of a single α locus, while the cistrons controlling the γ, μ, and α immunoglobulin classes are nonallelic and present in all rabbits, these findings imply that the rabbit heavy chain is controlled by at least *two separate germ-line genes.*

One gene, V-H-, codes for the N-terminal variable region in which the *a* allotypic markers are located, and a second gene, either C_γ, C_μ, or C_α, codes for the constant C-terminal region. (For a schematic illustration, see Fig. 1.) The fusion which eventually produces a single polypeptide heavy chain presumably occurs at the DNA or mRNA level since the experiments of Fleischman[26] indicate that the rabbit γ chain has a single growing point. Whatever the mechanism of fusion, the two-gene explanation requires a common recognition site at the ends of all the $C_{(H)}$ genes to which the same $V_{(H)}$ gene can be joined. This prediction can be tested by examining the relevant portion of the various heavy chains for lengths of identical sequences. The existence of identical sequences in these portions is indicated by the common binding of the light chains to all three classes of heavy chains.

The question whether the heavy chains from other species are also controlled by two separate germ-line genes cannot be resolved until genetic markers have been located in the variable regions. However, indirect supporting evidence is

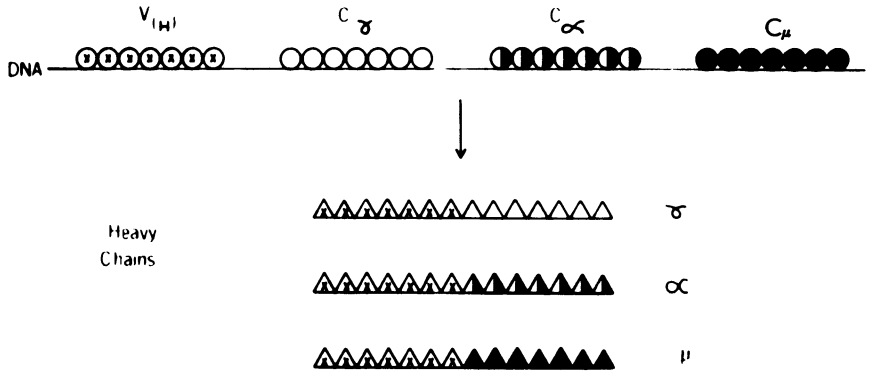

FIG. 1. Schematic representation of the 2 gene control of antibody heavy chains. Circles represent nucleotide triplets in DNA; triangles represent amino acid residues in heavy chains.

available from structural studies of human myeloma heavy chains. The amino acid sequences of the N-terminal peptides were found to be highly variable but did not appear to be specific for the class of heavy chain from which the peptides were derived.[27-30] These results are consistent with the independent genetic control of the variable and constant regions.

The only evidence contrary to the two-gene hypothesis is the report by Prahl and Porter[31] that the a3 allotype is correlated with a methionine-threonine interchange at about position 220 in the constant region of γ chains. However, this methionine substitution was not observed in similar studies in our laboratory using a different source of homozygous a3b4 rabbits, and more recent work by Prahl *et al.*[32] suggests that the methionine replacement is associated with an allotypic specificity, a11, of the constant region rather than with the a3 allotype of the variable region.

The identity of the specificity amino acid replacements in the different heavy chains of arsonic or lac antibody is consistent with a somatic mechanism for generating antibodies from a variable region gene which is separate from the constant region genes. According to this model, the many genes producing antibody sequences are evolved during the somatic development of the immune system from a single parent $V_{(H)}$ in the germ line. Each variable gene descendant is segregated in an individual cell where it can be linked to the $C_{(H)}$ gene of any class. The identity of the specificity residues results from the sharing of the same variable region genes defining a particular antibody by the $C_{(H)}$ genes of all classes. Furthermore, the results imply that clonal selection by the antigen either precedes or is not affected by the particular $C_{(H)}$ gene linkage.

Thus, both the results of the allotypic markers and the specificity differences indicate that at least two genes control the heavy chains of antibodies. The mechanism of antibody biosynthesis would appear, therefore, to differ from that of enzymes and proteins such as hemoglobin whose genetic characteristics have been elucidated. It is conceivable that other vertebrate proteins, i.e., the histones,[33] may also show a mechanism similar to that demonstrated for antibody heavy chains. There are evident evolutionary advantages to multiple gene control of polypeptide chains. In the case of antibodies, information for the rec-

ognition of a specific antigenic determinant is combined with the information for the general biological properties of antibodies, complement fixation, fixation to skin, passage through membranes, etc.

The data in this paper do not preclude the possibility that more than two genes function. The finding that the specificity differences occur independently of the allotype of the heavy chain suggests that there may in fact be *three*, instead of two, nonallelic genes which contribute to a single, polypeptide chain product. One gene would produce the portion associated with the allotypic markers, a second would produce the variable region associated with combining specificity, and a third would produce the constant portion associated with class specificity. Studies to evaluate these alternatives are in progress.

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